

AD-A220 029

1. REPORT SECURITY CLASSIFICATION * Unclassified			10. RESTRICTIVE MARKINGS		
2a. SECURITY CLASSIFICATION AUTHORITY			3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution is unlimited		
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE			4. PERFORMING ORGANIZATION REPORT NUMBER(S) NMRI 90-10		
6a. NAME OF PERFORMING ORGANIZATION Naval Medical Research		6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION Naval Medical Command		
6c. ADDRESS (City, State, and ZIP Code) Bethesda, Maryland 20814-5055			7b. ADDRESS (City, State, and ZIP Code) Department of the Navy Washington, D.C. 20372-5120		
8a. NAME OF FUNDING/SPONSORING ORGANIZATION Naval Medical Research and Development Command		8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER		
c ADDRESS (City, State, and ZIP Code) Bethesda, Maryland 20814-5055			10. SOURCE OF FUNDING NUMBERS		
			PROGRAM ELEMENT NO. 63706	PROJECT NO. M0095.003	TASK NO. 1007
			WORK UNIT ACCESSION NO DN677130		
11. TITLE (Include Security Classification) Monovalent ligands of complement receptor 2 inhibit whereas polyvalent ligands enhance anti-Ig-induced human B cell intracytoplasmic free calcium concentration					
12. PERSONAL AUTHOR(S) Tsokos GC, Lambris JD, Finkelman FD, Anastassiou ED, June CH					
13a. TYPE OF REPORT journal article	13b. TIME COVERED FROM _____ TO _____		14. DATE OF REPORT (Year, Month, Day) 1990	15. PAGE COUNT 6	
16. SUPPLEMENTARY NOTATION Reprinted from: The Journal of Immunology 1990 March 1; Vol.144 No.5 pp 1640-1645					
17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number) B cell; Complement receptor 2; Antigen receptor; cell calcium		
FIELD	GROUP	SUB-GROUP			
19. ABSTRACT (Continue on reverse if necessary and identify by block number)					
<p style="text-align: center;">DTIC ELECTE APR 02 1990</p> <p style="text-align: center;">S E D</p> <p style="text-align: center;">(60)</p>					
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT <input checked="" type="checkbox"/> UNCLASSIFIED/UNLIMITED <input type="checkbox"/> SAME AS RPT. <input type="checkbox"/> DTIC USERS			21. ABSTRACT SECURITY CLASSIFICATION Unclassified		
22a. NAME OF RESPONSIBLE INDIVIDUAL Phyllis Blum, Information Services Division			22b. TELEPHONE (Include Area Code) 202-295-2188		22c. OFFICE SYMBOL ISD/ADMIN/NMRI

DD FORM 1473, 84 MAR

83 APR edition may be used until exhausted.
All other editions are obsolete.SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

A-120

Accession for
 NTIS GRA&I
 DTIC TAB
 Unannounced
 Justification

By _____
 Distribution/
 Availability Code

Dist	Avail and/or Special
------	-------------------------

MONOVALENT LIGANDS OF COMPLEMENT RECEPTOR 2 INHIBIT WHEREAS POLYVALENT LIGANDS ENHANCE ANTI-Ig-INDUCED HUMAN B CELL INTRACYTOPLASMIC FREE CALCIUM CONCENTRATION¹

GEORGE C. TSOKOS,^{2*} JOHN D. LAMBRIS,³ FRED D. FINKELMAN,^{*}
EVANGELOS D. ANASTASSIOU,⁴ AND CARL H. JUNE⁵

From the ¹Department of Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD 20814; ²Kidney Diseases Section, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892; ³Basel Institute for Immunology, 4005 Basel, Switzerland; ⁴Naval Medical Research Institute, Bethesda, MD 20814

We have performed experiments to investigate the role of ligands for complement receptor 2 (CR2) in human B cell activation. Flow microfluorimetry was used to assess changes in free intracytoplasmic calcium concentration [Ca^{2+}] in indo-loaded B cells, immediately after exposure to anti- μ antibody and to monovalent or polyvalent CR2 ligands. As monovalent ligands we used the C3d fragment and synthetic C3 peptides (peptides P14, residues 1201-1214, and P28, residues 1187-1214). As polyvalent ligands we used i) an intact monoclonal mouse anti-CR2 antibody (HB5) and its F(ab')₂ fragment, ii) tetravalent P13 ((residues 1202-1214) 4-template), and iii) P28 conjugated to BSA (molar ratio 5/1). Anti-CR2 antibody HB5, tetravalent P13, and P28 conjugated to BSA, enhanced the ability of F(ab')₂ fragments of the IgG fraction of goat anti-human μ antibody to increase human B cell [Ca^{2+}]_i. In contrast, the monomeric CR2 ligands C3d and P28 inhibited the anti- μ -induced increase in human B cell [Ca^{2+}]_i. Multivalent P13, P28, and the HB5, by themselves, did not affect B cell [Ca^{2+}]_i. These experiments suggest that the valence of the CR2 ligands is crucial for the nature (synergistic vs antagonistic) of the message transmitted through the CR2.

CR2³ is a 140-kDa glycoprotein (1-3) that specifically binds iC3b, C3dg, and C3d fragments of C3 (4, 5) and the EBV envelope protein gp350/220 (6, 7). CR2 is expressed primarily by B cells, although it has been found on other cell types (5, 8).

CR2 binds a site on C3 that is composed of residues 1205-1214 of the C3 sequence (9). Synthetic C3 peptides P14 (residues 1201-1214) and P28 (residues 1187-1214) bind to CR2 (9, 10) and can be used as ligands in functional assays. A number of murine mAb that bind human

CR2, including the murine mAb HB5, have been produced (4, 8).

CR2 has been considered to play a role in B cell differentiation and proliferation. Polyclonal and monoclonal antibodies to CR2 (11-13) as well as particle bound C3d (14) have been shown to enhance B cell responses in different systems. More recently, HB5 has been found to act synergistically with μ -chain-specific antibody to increase B cell intracytoplasmic free calcium ($[\text{Ca}^{2+}]_i$) (15). Monomeric fluid phase C3d, in contrast, has been found to inhibit murine B cell proliferation (14). P14 and P28 peptides inhibit the maturation of murine B cell progenitors (16) while they support the growth of CR2-binding EBV lymphoblastoid B cell lines (10).

Cross-linking of B cell surface Ig induces hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C to inositol trisphosphate and diacylglycerol. Inositol trisphosphate causes the release of intracellular calcium from endoplasmic reticulum (17-21). [Ca^{2+}]_i can be accurately measured in isolated B cells using the Ca^{2+} -dependent indicator indo-1 (21, 22).

In this study we used the anti- μ -induced increase in [Ca^{2+}]_i, an early event in B cell activation, to study the role of CR2 in B cell function. We found that CR2-mediated modulation of B cell activation is ligand-valency-dependent, in that monomeric ligands inhibit the anti- μ -induced increase in [Ca^{2+}]_i whereas polyvalent CR2 ligands enhance the ability of anti- μ to increase [Ca^{2+}]_i.

MATERIALS AND METHODS

Cells. Peripheral blood MNC were obtained from heparinized blood by Ficoll-Hypaque density centrifugation. Human spleen cells were obtained through the Tissue Procurement Service of the Clinical Center of the National Institutes of Health.

Reagents. Affinity-purified F(ab')₂ fragments of the IgG fraction of goat anti-human μ (purchased from Jackson ImmunoResearch Laboratories, West Grove, PA) were used to stimulate B cells. PE-labeled monoclonal mouse IgG1k anti-human CD20 antibody was purchased from Becton Dickinson (Mountain View, CA). PE-labeled Leu-4, Leu-M3, Leu-7, and Leu-11 (Becton Dickinson) were used in some experiments. HB5 mouse IgG2ak anti-CR2 was obtained from the American Type Culture Collection, Rockville, MD. F(ab')₂ fragments of HB5 were prepared by pepsin digestion and separated from undigested IgG2ak molecules by absorption with protein A Sepharose (Pharmacia, Piscataway, NJ). Purity was demonstrated by SDS-PAGE analysis. F(ab')₂ fragments of the IgG fraction of goat anti-murine IgG antibody were purchased from Cappel-Cooper Biomedicals (Malvern, PA). F(ab')₂ fragments of goat anti-human- μ and/or HB5 antibodies were bound onto cyanogen bromide-activated Sepharose (Pharmacia) according to the provided instructions.

C3 was prepared from human plasma and C3d was prepared by digestion as previously described (23). C3-derived CR2-binding pep-

Received for publication July 5, 1989.

Accepted for publication November 29, 1989.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹This work was supported in part by Uniformed Services University of the Health Sciences Grant C083BJ. The Basel Institute of Immunology was founded and is entirely supported by F. Hoffmann-LaRoche Ltd. Co., Basel, Switzerland.

*Address correspondence and reprint requests to Dr. George C. Tsokos, NIH, Bldg. 10, Rm. 3N112, Bethesda, MD 20892.

²Abbreviations used in this paper: CR, complement receptor; [Ca^{2+}]_i, intracytoplasmic free calcium concentration; MNC, mononuclear cells; PE, phycoerythrin.

tides (P14 and P28) were prepared as previously detailed (9, 10). Both bind to CR2 but P28 binds with a higher affinity than does P14 (9). The P13-template was synthesized according to the principle of Mutter et al. (24) as previously detailed (10). In brief, the amino acid template was synthesized first on a resin by using butyloxycarbonyl-Lys(F-moc)-OH for four cycles. Four P13 peptides were synthesized on the template-resin after deprotection of the F-moc protected sites. The final product was cleaved from the resin and purified by gel filtration. It was calculated that the majority (>90%) of the CR2 were occupied when P28 was used at concentrations greater than 60 $\mu\text{g}/\text{ml}$ in cell suspensions of $1 \times 10^6/\text{ml}$ (10). P28, to which a cysteine residue was added to the amino-terminus, was conjugated to BSA as described by Lerner et al. (25). The P28:BSA molar ratio was calculated to be 5:1.

Intracellular ionized calcium assay. $[\text{Ca}^{2+}]_i$ in single cells was measured as described (26, 27). Briefly, cells were loaded with the acetoxymethyl ester of indo-1 (Molecular Probes, Eugene, OR). Initial concentrations of 3 μM of this compound, resulted in an intracellular indo-1 concentration of 20 to 50 μM . After the loading procedure the cells were washed, placed in fresh medium at $2.5 \times 10^6/\text{ml}$ and stored in the dark at room temperature until analysis. Immediately before each assay, indo-1-loaded cells were diluted to $1 \times 10^6/\text{ml}$ with medium (HBSS, containing 1 mM CaCl_2 , 1 mM MgCl_2 , 20 mM HEPES, and 10% FCS), equilibrated at 37°C and analyzed by flow cytometry at 400 to 500 cells/s. For each cell analyzed the ratio of violet:blue fluorescence was digitally calculated in real time. The $[\text{Ca}^{2+}]_i$ (nM) was calculated from the indo-1 violet to blue ratio after proper calibrations were performed as previously detailed (27). Before loading with indo-1 the cells were stained with PE-labeled anti-CD20 or with a mixture of PE-labeled anti-Leu-4, Leu-M3, Leu-7, and Leu-11 by incubating for 30 min at 4°C. This permitted the identification (gating) of B cells by positive or negative selection, respectively. Preliminary experiments demonstrated that prior staining of cells with anti-CD20 antibody had no effect on the anti- μ -induced increase in $[\text{Ca}^{2+}]_i$.

Proliferation assays. B cells (70 to 80% CD20 $^+$) were enriched from peripheral blood or spleen by depletion of monocytes through plastic adherence and T cells through sheep erythrocyte rosetting and were cultured in 96-well round-bottom microtiter plates at 50,000/well in medium RPMI 1640 containing 10% FCS. Cells were harvested 72 h after the initiation of the culture and were pulsed with 0.5 $\mu\text{Ci}/\text{well}$ of ^3H -thymidine for 18 h before harvesting.

RESULTS

Cross-linking of IgM with $F(ab')_2$ fragments of goat anti-IgM antibody leads to a dose-dependent increase in $[\text{Ca}^{2+}]_i$. In a typical experiment (Fig. 1) peripheral blood MNC were stained first with PE-labeled anti-CD20 and then loaded with indo-1. They were equilibrated at 37°C before use and exposed to graded doses of $F(ab')_2$ goat

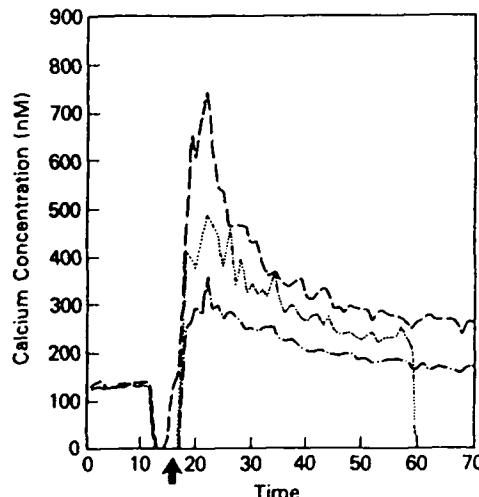


Figure 1. Peripheral MNC were first stained with PE-anti-CD20, loaded with indo-1 and equilibrated to 37°C before analysis. $F(ab')_2$ -goat anti-human μ was added to cells at the indicated time (arrow) at different concentrations: (—), 3 $\mu\text{g}/\text{ml}$; (---), 10 $\mu\text{g}/\text{ml}$; (- - -), 30 $\mu\text{g}/\text{ml}$. The interval 0–100 on the abscissa is equivalent to 6 min. The concentration of Ca^{2+} was calculated as described in Materials and Methods and is expressed as nM.

anti-human μ . $[\text{Ca}^{2+}]_i$ was observed to increase instantly and decline and plateau a few minutes later; similar responses were obtained from MNC from 14 different normal individuals and four different spleen B cell preparations that were studied during the course of these experiments. Peripheral cells were stained first with a mixture of PE-labeled anti-leu 4, leu-M3, leu-7 and leu-11, (negative selection) or with anti-CD20 (positive selection) before loading with indo-1 and analysis. Positively or negatively gated cells responded similarly to anti- μ .

To investigate the effect of monovalent CR2 ligands on the anti- μ -induced increase of $[\text{Ca}^{2+}]_i$ we first exposed the cells to graded concentrations of C3d, the natural ligand of CR2, and then added anti- μ antibody. As shown in Figure 2, preincubation of cells with C3d for 5 min at 37°C inhibits the ability of 10 $\mu\text{g}/\text{ml}$ of anti- μ antibody to increase $[\text{Ca}^{2+}]_i$. In four more experiments, which were performed with either peripheral MNC (twice) or spleen cells (twice), C3d (60 $\mu\text{g}/\text{ml}$) inhibited the anti- μ -induced $[\text{Ca}^{2+}]_i$ response by 40 to 62%. C3d inhibition of the B cell $[\text{Ca}^{2+}]_i$ response was greatest when the anti- μ antibody was used at 10 $\mu\text{g}/\text{ml}$ and less at higher and lower anti- μ concentrations (Table I). Preincubation of the cells with C3d for longer periods of time (up to 20 min), inhibited the anti- μ response to the same extent. Addition of C3d, simultaneously with the anti- μ antibody, had a less reproducible inhibitory effect. Addition of C3d alone at the time of the initiation of the analysis had no effect on B

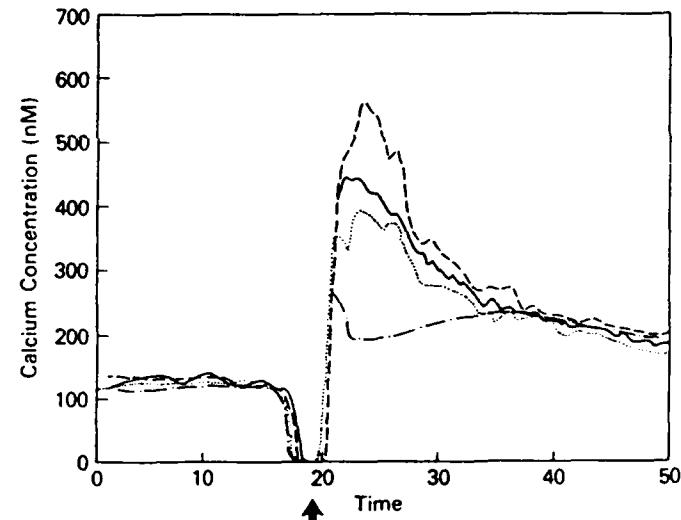


Figure 2. Inhibition of the anti- μ -induced increase in B cell $[\text{Ca}^{2+}]_i$ by C3d. Peripheral MNC were stained with PE-anti-CD20, loaded with indo-1 and equilibrated at 37°C before analysis. They were treated with 10 $\mu\text{g}/\text{ml}$ (—), 30 $\mu\text{g}/\text{ml}$ (---), or 60 $\mu\text{g}/\text{ml}$ (- - -) of C3d, or were left untreated (----) before the addition of 10 $\mu\text{g}/\text{ml}$ of $F(ab')_2$ -goat anti- μ .

TABLE I
Inhibition of anti- μ induced increase in $[\text{Ca}^{2+}]_i$ of B cell-enriched peripheral blood MNC by the CR2 ligand P28

	Concentration of $F(ab')_2$ Anti- μ Antibody ($\mu\text{g}/\text{ml}$)		
	3	10	30
No ligand	309 ^a	455	570
60 $\mu\text{g}/\text{ml}$ C3d	230 (44)	260 (60)	455 (26)
100 $\mu\text{g}/\text{ml}$ P28	280 (16)	270 (56)	470 (23)

^a Maximum recorded channel $[\text{Ca}^{2+}]_i$ (nM). B cell-enriched peripheral blood MNC were incubated with or without 100 $\mu\text{g}/\text{ml}$ of P28 for 5 min at 37°C before stimulation with the indicated concentrations of anti- μ antibody. Numbers in parentheses indicate the % of inhibition that was calculated after subtracting the baseline $[\text{Ca}^{2+}]_i$ (130 nM).

cell $[Ca^{2+}]_i$ (Fig. 3).

The above experiment was repeated, substituting for C3d the peptides P14 (residues 1201-1214 of C3) or P28 (residues 1187-1214 of C3). Preincubation of cells with P14 had little effect on the anti- μ -induced increase in $[Ca^{2+}]_i$ (Fig. 4). Prolongation of this preincubation time up to 30 min still had little effect. Similarly, P14 failed to inhibit the anti- μ -induced human spleen B cell $[Ca^{2+}]_i$ response (data not shown). In contrast, preincubation of cells for 5 min at 37°C with graded doses of P28, which is bound by CR2 with higher affinity than P14 (9), inhibited the anti- μ -induced increases of $[Ca^{2+}]_i$ in a dose-dependent manner (Fig. 5). P28 (100 μ g/ml) inhibited the anti- μ -induced $[Ca^{2+}]_i$ response in six more experiments

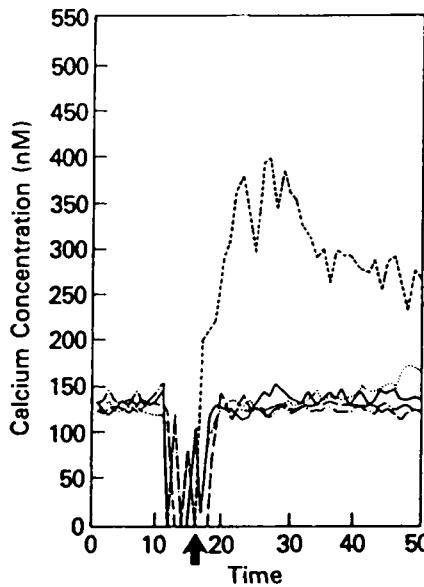


Figure 3. Monovalent or polyvalent CR2 ligands have no effect on the B cell $[Ca^{2+}]_i$ when present in the absence of anti- μ antibody. Peripheral MNC were treated as in the legend to Figure 2. After equilibration at 37°C they were treated with (—), 50 μ M/ml; or (----), 100 μ g/ml of P28, or (···), no P28, for 5 min before the addition of 10 μ g/ml of F(ab')₂-goat anti- μ (arrow).

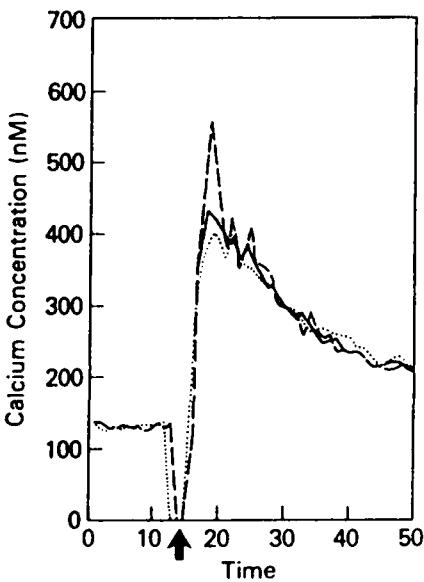


Figure 4. The CR2-binding peptide P14 fails to inhibit the anti- μ -induced increase in $[Ca^{2+}]_i$. Peripheral MNC were treated as described in the legend to Figure 2. After equilibration at 37°C the cells were treated with (—), 50 μ M/ml; or (----), 100 μ g/ml of P14, or P28 (···) before exposure to 10 μ g/ml of F(ab')₂-goat anti- μ (arrow).

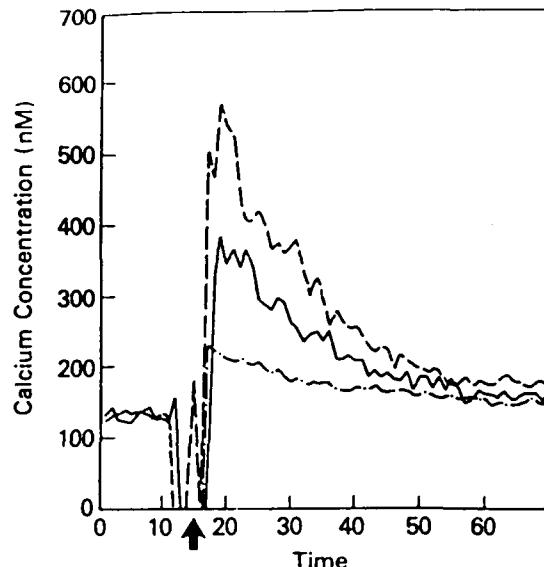


Figure 5. The CR2-binding peptide P28 inhibits the anti- μ -induced increase in $[Ca^{2+}]_i$. Peripheral MNC were treated as in the legend to Figure 2. After equilibration at 37°C they were treated with (—), 50 μ M/ml; or (----), 100 μ g/ml of P28, or (···), no P28, for 5 min before the addition of 10 μ g/ml of F(ab')₂-goat anti- μ (arrow).

using peripheral MNC and 2 more using spleen cells; the percentage of inhibition ranged from 45 to 65%. As shown in Table I, P28 inhibited optimally the $[Ca^{2+}]_i$ response, when the anti- μ antibody was used at 10 μ g/ml and to a lesser extent when higher or lower concentrations were used (Table I). Addition of either P14 or P28 at the initiation of the analysis, in the absence of anti- μ antibody, had no effect on $[Ca^{2+}]_i$ (Fig. 3). These experiments show clearly that monovalent CR2 ligands inhibit an early, anti- μ -induced, B cell activation event, the increase of $[Ca^{2+}]_i$.

Because polyvalent CR2 ligands have been reported to enhance B cell responses we next investigated the effect of exposure of human peripheral blood and spleen B cells to polyvalent CR2 ligands on their $[Ca^{2+}]_i$. As such we have used HB5 anti-CR2 antibody, F(ab')₂, fragments of the same antibody, a synthetic tetravalent CR2 ligand: the (P13)₄-template, and a pentavalent P28-BSA conjugate. Exposure of cells to intact HB5 for 5 min at 37°C (or for up to 20 min) before addition of 3 μ g/ml of the F(ab')₂ fragment of anti-human μ antibody clearly enhanced the increase of $[Ca^{2+}]_i$. The increase was dose-dependent and the effect was reproduced in both positively and negatively selected peripheral blood (five experiments) and spleen B cells (two experiments). Optimal enhancement was observed when suboptimal concentrations (3 μ g/ml) of anti- μ antibody were used. Because cross-linking of B cell membrane Ig with the B cell FcR inhibits B cell activation (22), we repeated this experiment with the F(ab')₂ fragment of HB5. Similar results were obtained (Fig. 6). HB5 alone had no effect on $[Ca^{2+}]_i$ (Fig. 3). To determine if a tetravalent ligand bound by CR2 would mimic the effect of the divalent HB5 antibody on the anti- μ -induced increase in $[Ca^{2+}]_i$, we studied the ability of the synthetic, tetravalent (P13)₄-template to affect the increase. Preincubation of cells with (P13)₄-template before addition of F(ab')₂ fragments of goat anti-human μ enhanced the anti- μ -induced $[Ca^{2+}]_i$ increase (Fig. 7). As was observed with HB5, (P13)₄-template had

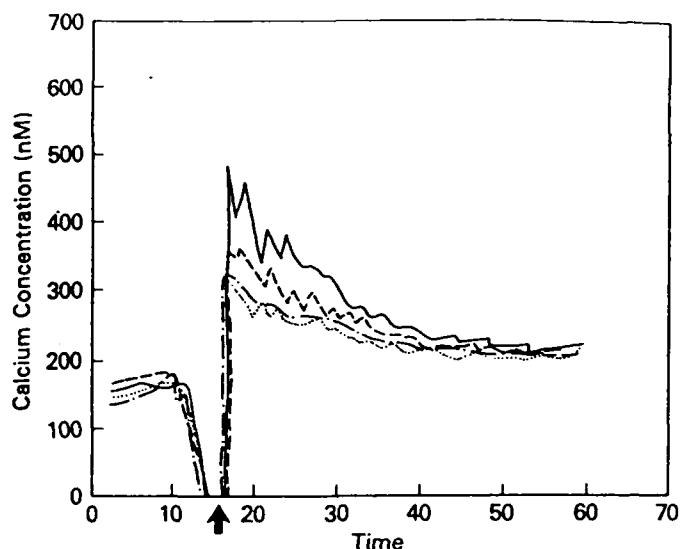


Figure 6. Effect of HB5 anti-CR2 antibody on the anti- μ induced increase in B cell $[Ca^{2+}]_i$. Peripheral MNC were treated as described in the legend to Figure 2, treated with 0.1 (···), 0.5 (---), or 1.0 (—) μ g/ml of $F(ab')_2$ fragment of HB5 or no HB5 (----) for 5 min, before exposure to 3 μ g/ml of $F(ab')_2$ goat anti- μ (arrow).

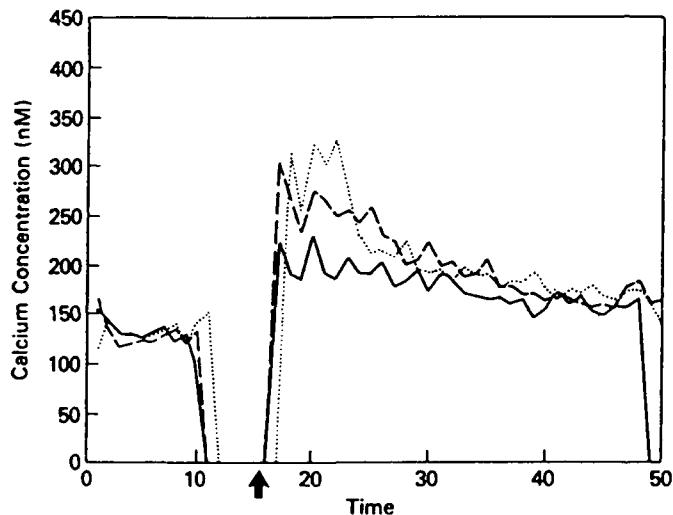


Figure 7. Effect of (P13)4-template on the anti- μ antibody induced increase in B cell $[Ca^{2+}]_i$. Peripheral MNC were treated as described in the legend to Figure 2. After equilibration at 37°C they were treated with 10 μ g/ml (---), 50 μ g/ml (···) or no (P13)4-template (—) for 5 min before adding 3 μ g/ml of $F(ab')_2$ goat anti- μ antibody.

no direct effect on B cell $[Ca^{2+}]_i$ (Fig. 3). Preincubation of B cell-enriched human peripheral B cells with P28 bound to BSA (molar ratio 5:1), for 5 min before addition of anti- μ antibody, also enhanced (in three different experiments) the anti- μ -induced increase in $[Ca^{2+}]_i$ (Table II). BSA alone had no effect on the anti- μ -induced increase in $[Ca^{2+}]_i$.

Finally, we asked whether the observed effects of CR2 ligands on the anti- μ antibody-induced increase in B cell $[Ca^{2+}]_i$ were associated with similar effects on anti- μ -induced B cell proliferation. B cell-enriched peripheral blood cells (70 to 80% CD20 $^+$ cells) were cultured with Sepharose-bound $F(ab')_2$ fragments of anti-human μ in the presence or absence of HB5 antibody. The presence of HB5, either in soluble form or coupled to Sepharose, had no effect on the anti- μ -induced proliferative response. In contrast, culture of B cell-enriched PBMC with Sepharose beads, to which both anti- μ and HB5 antibod-

TABLE II
Effects of P28 and P28 conjugated to BSA on the anti- μ -induced increase in human B cell $[Ca^{2+}]_i$

	Concentration of $F(ab')_2$ Anti- μ Antibody (μ g/ml)	
	3	10
No ligand	231*	310
100 μ g/ml P28	205	210
P28 conjugated to BSA (μ g/ml)		
10	309	396
50	395	424
100	456	465

* Maximum recorded channel $[Ca^{2+}]_i$ (nM); baseline $[Ca^{2+}]_i$ was 130 nM. B cell enriched (76%) CD20 $^+$ peripheral blood MNC were incubated with the indicated ligands for 5 min before stimulation with the indicated concentrations of anti- μ antibody. P28 or P28 conjugated to BSA did not increase $[Ca^{2+}]_i$ above 140 nM.

TABLE III
HB-5 enhances anti- μ -induced B cell proliferation

	cpm*
Sepharose-bound goat IgG ^b	890 ± 75
Sepharose-bound $F(ab')_2$ a- μ	13,950 ± 480
Sepharose-bound HB5	1,850 ± 115
Sepharose-bound $F(ab')_2$ a- μ + Sepha- rose-bound HB5	12,890 ± 560
Sepharose-bound HB5 and $F(ab')_2$ goat- μ	21,560 ± 850

* 50,000 cells (75% CD20 $^+$)/200 μ l of medium/well were cultured in 96-well plates, pulsed with 0.5 μ Ci 3 H-thymidine/well for 18 h and harvested after 72 h of culture.

^a All Ig were bound to activated Sepharose at a concentration of 1 mg/ml of gel. Five μ l of Sepharose-bound antibody were added to each ml of culture medium.

TABLE IV
Effect of C3d and P28 on anti- μ antibody induced B cell proliferation

	0	+0	650 ± 65
10 μ g/ml $F(ab')_2$ goat anti- μ	+0	8,750 ± 470	
-	+10 μ g/ml C3d	8,970 ± 380	
-	+50 μ g/ml C3d	5,480 ± 350	
-	+100 μ g/ml C3d	2,350 ± 115	
-	+10 μ g/ml P28	9,150 ± 350	
-	+50 μ g/ml P28	5,730 ± 310	
-	+100 μ g/ml P28	1,250 ± 65	

* Cultures were performed as described in Table II.

ies had been coupled, stimulated more DNA synthesis than did Sepharose beads that bore anti- μ , but not HB5 (Table III). Culture of B cell-enriched peripheral blood cells with soluble HB5 in the presence of 3 μ g/ml of soluble anti- μ failed to induce detectable DNA synthesis, and soluble HB5 had no effect on DNA synthesis induced by 10 μ g/ml of soluble $F(ab')_2$ of anti- μ . This was not surprising inasmuch as HB5 had little effect on the increase of $[Ca^{2+}]_i$ that is stimulated by this concentration of anti- μ antibody.

Monovalent CR2 ligands had a clear inhibitory effect on the induction of B cell DNA synthesis by soluble $F(ab')_2$ fragments of anti- μ antibody. When B cell-enriched PBMC were cultured with soluble $F(ab')_2$ anti- μ antibody in the presence of graded doses of P28 or C3d, B cell proliferative responses were inhibited in a dose-dependent manner (Table IV). Thus, monovalent CR2 ligands inhibit both the induction of increased B cell $[Ca^{2+}]_i$ and DNA synthesis by mitogenic concentrations of anti- μ antibody, whereas divalent or polyvalent CR2 ligands enhance the ability of submitogenic concentrations of anti- μ antibody to stimulate an increase in B cell $[Ca^{2+}]_i$ but do not stimulate mitogenesis. Soluble divalent CR2 ligands have little effect on enhancement of B cell $[Ca^{2+}]_i$.

or DNA synthesis by mitogenic concentrations of anti- μ antibody and particulate, polyvalent CR2 ligands, whose effects on B cell $[Ca^{2+}]_i$ are difficult to measure, only enhance the ability of anti- μ antibody to induce B cell DNA synthesis when they are associated with anti- μ in the same particle.

DISCUSSION

In the present study we have used flow microfluorimetry to study the effect of monovalent CR2 ligands or ligands capable of cross-linking CR2 on the ability of anti- μ antibody to activate B lymphocytes. Because of variability in earlier reports of the effects of CR2 ligands on B cell proliferation and differentiation (11–15, 28) we considered that the valency of the CR2 ligands might be important in determining the CR2-transduced signal. Hence, we used clearly defined monovalent, divalent, tetravalent, and pentavalent CR2 ligands. None of these ligands had any effect on $[Ca^{2+}]_i$ if added alone at the initiation of the analysis.

Occupancy of CR2 by monovalent ligands before stimulation of B cells with anti- μ antibody clearly inhibits B cell activation. In our study, monomeric C3d, a natural ligand of CR2, as well as the synthetic peptide P28, which represents the CR2 binding sequence (residues 1187–1214) of C3, inhibited the anti- μ induced $[Ca^{2+}]_i$ increase. P14, another synthetic peptide (residues 1201–1214) that also binds to CR2 but with lesser affinity (9), failed to inhibit significantly the anti- μ -induced B cell $[Ca^{2+}]_i$ response. In previous reports, monomeric CR2 ligands have been found to inhibit murine B cell progenitor (16) and lymphoblastoid B cell growth (10). Similarly, CR1 monomeric ligands inhibit B cell differentiation (29) and macrophage Ia antigen expression (30).

In contrast to the effects of monovalent CR2 ligands, polyclonal anti-CR2 F(ab')₂ (11), monoclonal anti-CR2 (12, 13) and glutaraldehyde bound C3d (14) enhance anti- μ antibody-induced B cell proliferation. Polymeric C3dg primes human B cells to proliferate more vigorously upon stimulation with anti- μ antibody (31). In this study we used F(ab')₂ fragments of HB5 to demonstrate that they enhance the ability of suboptimal concentrations of anti- μ antibody to induce an increase in $[Ca^{2+}]_i$. This finding is in agreement with a recent report (15). We extended this observation by demonstrating that a synthetic tetravalent CR2 ligand ((P13)₄-template), and P28 conjugated to BSA (pentavalent) similarly enhance the anti- μ antibody-induced increase in B cell $[Ca^{2+}]_i$.

Our study thus demonstrates that the valence of the CR2 ligand determines whether it inhibits or enhances the early activation of B cells by anti- μ antibody. The mechanism by which CR2 ligands, depending on their valence, either inhibit or enhance anti- μ antibody-induced increases in $[Ca^{2+}]_i$ is not established. It is possible that the occupancy of CR2 by a univalent ligand directly generates a signal that inhibits anti- μ -induced phospholipase C activation (32, 33), whereas the cross-linking of CR2 directly generates a different signal that has the opposite effect. An alternative possibility is that CR2-ligand interactions, rather than directly generating signals, affect anti- μ antibody induction of increased B cell $[Ca^{2+}]_i$ by modifying the ability of a B cell membrane Ig-ligand interaction to activate phospholipase C. Because such activation requires the cross-linking of membrane

Ig, and because membrane Ig, when cross-linked, becomes associated with ligand-occupied CR2 (34), it is possible that cross-linking of CR2 by divalent or polyvalent ligands promotes the formation of domains of membrane Ig that have the conformation required to activate phospholipase C. Such promotion of membrane Ig cross linking by an interaction between CR2 and a polyvalent ligand would be particularly marked when the ligands for membrane Ig and CR2 were physically associated. Notably, we and others (15) have been best able to demonstrate synergistic induction of B cell DNA synthesis by anti-Ig and anti-CR2 antibodies when these two antibodies have been directly associated. In contrast to its promotion of membrane Ig cross-linking when occupied by a multivalent ligand, CR2, when occupied by a univalent ligand, might act as a brake on the cross-linking of associated membrane Ig rather than contribute to its cross-linking. This could interfere with the establishment of cross-linked membrane Ig domains that are capable of activating phospholipase C, with the direct result that the increases in $[Ca^{2+}]_i$ and protein kinase C activity are inhibited. This, in turn could limit the induction of a late event in B cell activation, such as DNA synthesis.

Regardless of the mechanism involved, the opposite effects of CR2 interactions with monovalent or multivalent ligands could have important physiologic effects on the development of humoral immune responses *in vivo*. Bacterial Ag that directly fix C by the alternative pathway would not only form Ag-C3 fragment complexes that could associate with multiple Ig and CR2 molecules on the surface of Ag-specific B cells, but would also release soluble, univalent C3 fragments. The multivalence of the C3 fragments on such a bacterium-C3 complex could contribute to activation of B cells specific for bacterial Ag, whereas univalent, circulating C3 fragments might inhibit the polyclonal activation of B cells that lack specificity for bacterial Ag. Thus, the opposite effects of monovalent and polyvalent CR2 ligands on B cell activation could provide a means by which the C system could simultaneously contribute to the generation of a specific antibody response and restrain the development of a polyclonal antibody response that would be drain on the immune system and could produce potentially harmful autoantibodies.

Acknowledgments. We are grateful to Dr. G. Thymronitis for his help with reagents, Mr. D. Smoot and Mr. D. Avila for expert technical assistance, and Mrs. Denise R. Giles for typing this manuscript.

REFERENCES

1. Barel, M., C. Charriaut, and R. Frade. 1981. Isolation and characterization of C3b receptor-like molecule from membranes of a human B lymphoblastoid cell line (Raji). *FEBS Lett.* 136:111.
2. Iida, K., L. Nadler, and V. Nussenzweig. 1983. Identification of the membrane receptor for the complement fragment C3d by means of a monoclonal antibody. *J. Exp. Med.* 158:1021.
3. Weis, J. J., T. F. Tedder, and D. T. Fearon. 1984. Identification of a 145,000 M_r membrane protein as the C3d receptor (CR2) of human lymphocytes. *Proc. Natl. Acad. Sci. USA* 81:881.
4. Lambris, J. D., and G. C. Tsokos. 1986. The biology and pathophysiology of complement receptors. *Cancer Res.* 46:515.
5. Lambris, J. D. 1988. The multifunctional role of C3, the third component of complement. *Immunol. Today* 9:387.
6. Tanner, J., J. Weis, D. F. Fearon, Y. Whang, and E. Kieff. 1987. Epstein-Barr virus gp350/220 binding to the B lymphocyte C3d receptor mediates adsorption, capping, and endocytosis. *Cell* 50:203.
7. Nemerow, G. R., C. Mold, V. K. Schwed, V. Tollefson, and N. R.

Cooper. 1987. Identification of gp350 as the viral glycoprotein mediating attachment of Epstein-Barr virus (EBV) to the EBV/C3d receptor of B cells: sequence homology of gp350 and C3 complement fragment C3d. *J. Virol.* 61:1416.

8. Ross, G. D., and M. E. Medof. 1985. Membrane complement receptors specific for bound fragments of C3. *Adv. Immunol.* 37:217.

9. Lambris, J. D., V. S. Ganu, S. Hirani, and H. J. Muller-Eberhard. 1985. Mapping of the C3d receptor (CR2)-binding site and a neoantigenic site in the C3d domain of the third component of complement. *Proc. Natl. Acad. Sci. USA* 82:4235.

10. Servis, C., and J. D. Lambris. 1989. C3 synthetic peptides support growth of human CR2-positive lymphoblastoid cells. *J. Immunol.* 142:2207.

11. Prade, R., M. C. Crevon, M. Barel, A. Vazquez, L. Krikorian, C. Charriaut, and P. Galanaud. 1985. Enhancement of human B cell proliferation by an antibody to the C3d receptor, the gp140 molecule. *Eur. J. Immunol.* 15:73.

12. Bohnsack, J. F., and N. R. Cooper. 1988. CR2 ligands modulate human B cell activation. *J. Immunol.* 141:2569.

13. Wilson, B., J. Platt, and N. E. Kay. 1985. Monoclonal antibodies to the 140,000 mol wt glycoprotein of B lymphocyte membranes (CR2 receptor) initiate proliferation of B cells *in vitro*. *Blood* 66:824.

14. Melchers, F., A. Erdel, T. Schulz, and M. P. Dierich. 1985. Growth control of activated, synchronized murine B cells by the C3d fragment of human complement. *Nature* 317:264.

15. Carter, R. H., M. O. Spycher, Y. C. Ng, R. Hoffman, and D. T. Fearon. 1988. Synergistic interaction between complement receptor type 2 (CD21) and membrane IgM on B lymphocytes. *J. Immunol.* 141:457.

16. Gisler, R. H., C. Schlienger, A. Soderberg, F. Lederman, and J. D. Lambris. 1988. Functional maturation of murine B lymphocyte precursors: II. of growth and differentiation. *Mol. Immunol.* 25:1113.

17. Harris, L. K., and J. C. Cambier. 1987. B lymphocyte activation: transmembrane signal transduction by membrane immunoglobulin in isolated cell membranes. *J. Immunol.* 139:963.

18. Bijsterbosch, M. K., J. C. Meade, G. A. Turner, and G. G. B. Klaus. 1985. B lymphocyte receptors and polyphosphoinositide degradation. *Cell* 41:999.

19. Ransom, J. T., L. K. Harris, and J. C. Cambier. 1986. Anti-Ig induces release of inositol 1,4,5-trisphosphate, which mediates mobilization of intracellular Ca⁺⁺ stores in B lymphocytes. *J. Immunol.* 137:708.

20. Coggeshall, K. M., and J. C. Cambier. 1984. B cell activation of phosphatidylinositol hydrolysis. *J. Immunol.* 133:3382.

21. Ransom, J. T., M. Chen, M. Sandoval, J. A. Posternak, D. Digiusto, and J. C. Cambier. 1988. Increased plasma membrane permeability to Ca⁺⁺ in anti-Ig-stimulated B lymphocytes is dependent on activation of phosphoinositide hydrolysis. *J. Immunol.* 140:3150.

22. Wilson, H. A., D. Greenblatt, C. W. Taylor, J. W. Putney, R. Y. Tsien, F. D. Finkelman, and T. M. Chused. 1987. The B lymphocyte calcium responses to anti-Ig is diminished by membrane immunoglobulin cross-linkage to the Fc_γ receptor. *J. Immunol.* 138:1712.

23. Lambris, J. D., N. J. Dobson, and G. D. Ross. 1980. Release of endogenous C3d inactivator from lymphocytes in response to triggering membrane receptors for $\beta 1H$ globulin. *J. Exp. Med.* 152:1625.

24. Mutter, M., E. Altmann, K. H. Altmann, R. Hersperger, P. Kozicj, K. Nebel, G. Tuchscherer, S. Builleumier, H. U. Gremlich, and K. Muller. 1988. The construction of new proteins. Artificial folding units by assembly of amphiphilic secondary structures on a template. *Helv. Chim. Acta* 71:835.

25. Lerner, R. A., N. Green, H. Alexander, F.-T. Liu, J. G. Sutcliffe, and T. M. Shinnick. 1981. Chemically synthesized peptides predicted from the nucleotide sequence of the hepatitis B virus genome elicit antibodies reactive with native envelope protein of Dane particles. *Proc. Natl. Acad. Sci. USA* 78:3403.

26. Rabinovitch, P. S., C. H. June, A. Grossman, and J. A. Ledbetter. 1986. Heterogeneity among T cells in intracellular free calcium responses after mitogen stimulation with PHA or anti-CD3. Simultaneous use of Indo-1 and immunofluorescence with flow cytometry. *J. Immunol.* 137:952.

27. June, C. H., P. S. Rabinovitch, and J. A. Ledbetter. 1987. CD5 antibodies increase intracellular ionized calcium concentration in T cells. *J. Immunol.* 138:2782.

28. Tedder, T. F., J. J. Weis, L. T. Clement, D. T. Fearon, and M. D. Cooper. 1981. The role of receptor for complement in the induction of polyclonal B-cell proliferation and differentiation. *J. Clin. Immunol.* 6:65.

29. Tsokos, G. C., M. Berger, and J. E. Balow. 1984. Modulation of human B cell immunoglobulin secretion by the C3b component of complement. *J. Immunol.* 132:622.

30. Mauer, V., R. Burger, D. Von Steldern, D. Bitter-Suermann, and V. Holding. 1984. Expression of Ia antigens on macrophages is reduced after stimulation with homologous C3d. *J. Immunol.* 132:2802. *Alcohol Clin Exp Res.* Vol. 14, No. 1, 1990: pp 130-133.

31. Carter, R. H., and D. T. Fearon. 1989. Polymeric C3dg primes human B lymphocytes for proliferation induced by anti-IgM. *J. Immunol.* 143:1755.

32. Bijsterbosch, M. K., and G. G. B. Klaus. 1985. Crosslinking of surface immunoglobulin and Fc receptor of B lymphocytes inhibits stimulation of inositol phospholipid breakdown via the antigen receptor. *J. Exp. Med.* 162:1825.

33. Rigley, K. P., M. M. Harnett, and G. G. B. Klaus. 1989. Cocrosslinking of surface immunoglobulin Fc receptors on B lymphocytes uncouples the antigen receptors from their associated G protein. *Eur. J. Immunol.* 19:481.

34. Tsokos, G. C., G. Thyphronitis, R. M. Jack, and F. D. Finkelman. 1988. Ligand-loaded, but not free complement receptors for C3d/C4b and C3d co-cap with cross-linked B cell surface IgM and IgD. *J. Immunol.* 141:1261.